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Long circulating drug carriers do accumulate in solid tumors, but do not specifically bind cancer cells. Thus, therapeutic drug delivery has limited efficacy and diagnostic imaging agents relatively low specificity and, for small metastases, low sensitivity. Substantial improvements could be achieved by the association of long-circulating drug carriers with "vector molecules" capable of binding specifically to cancer cells. Our proposal is based on the idea of using a genetic selection/screening technique to identify peptides that selectively recognize breast cancer cells (as opposed to normal cells of the same or different type). Such cancer-specific peptides will then be coupled to fleximer-based long-circulating drug carriers to confer upon these compounds the desired specificity. In the first year of our work, we have optimized conditions and screened three different phage display libraries for peptides that selectively bind to Erb2, an important cell surface receptor overexpressed in breast cancer cells. We have confirmed that the phage display technology can be used to identify peptides that bind to proteins expressed on the surface of breast cancer cells. The challenge for the upcoming year will be to identify peptides that retain specificity of binding when separated from the phage and tested in a free form in solution and/or coupled to a macromolecular carrier.

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L. Paul Patt 7/30/98

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Introduction

Macromolecular drug carriers provide one of the most promising approaches to improve delivery of therapeutic and diagnostic drugs to cancer cells. A very significant achievement in this area relates to the recent development of "long-circulating" macromolecular and colloidal preparations (polymers, micelles, liposomes, etc.). Clearance of these compounds from the blood stream and uptake by the reticuloendothelial system (RES) are very slow. As a consequence, macromolecular drug carriers circulate long enough to extravasate into tumors via "leaky" endothelium, and accumulate at these sites as a result of deficient lymphatic drainage, non-specific tissue binding and spontaneous endocytosis.

Although long circulating drug carriers do accumulate in solid tumors, they do not specifically bind cancer cells. Thus, the microdistribution of the drug within the tumor tissue may be suboptimal. Also, the drug complex can partially return to the blood stream by a reverse of the extravasation process through the leaky endothelium, or lymphatic drainage, or both. These limitations have two major consequences: 1) therapeutic drug delivery has limited efficacy, so that the therapeutic index remains relatively low; 2) diagnostic imaging agents for tumor staging have low specificity and, for small metastases, low sensitivity. Substantial improvements could be achieved by the association of long-circulating drug carriers with "vector molecules" capable of binding specifically to cancer cells. These molecules would (a) direct carrier binding specifically to cancer cells, minimizing nonspecific uptake by the surrounding tissue; and (b) prevent the carrier from exiting the tumor, and thus maintaining higher local drug concentrations. Previous attempts to develop cancer-specific vector molecules were based mostly on antibodies against tumor-specific antigens. This approach, however, has several limitations, related to the large size of the antibody molecules, and the fact that cancer-specific antigens are usually expressed at low concentrations, and only in limited subsets of cancer cells.

Our proposal is based on the idea of using a genetic selection/screening technique to identify peptides that selectively recognize breast cancer cells (as opposed to normal cells of the same or different type). Such cancer-specific peptides will then be coupled to fleximer-based long-circulating drug carriers to confer upon these compounds the desired specificity.

Body of Work

1) Assumptions, Experimental Approach and Results.

One frequent genetic alteration connected with breast cancer development is amplification and/or overexpression of the Erb2 oncogene. In order to optimize our selection/screening procedure for breast-cancer specific peptides, we decided at first to focus on the identification of peptides that can selectively bind to the extracellualr portion of the Erb2 protein. We were prompted by several reasons. First, by knowing what the specific target of the selected peptides would be, it would be possible to measure affinity of binding of the peptide-expressing phage, as well as of the biochemically synthesized peptide, either free in solution or coupled to a macromolecular carrier. This in turn would have allowed us to optimize conditions for additional studies with peptides binding to unidentified targets. Second, we made a collaborative arrangement with Dr. Kermit Carraway at Harvard Medical School, who provided us with SF9 insect cells overexpressing the Erb2 protein in a functional state on their surface. Use of normal SF9 cells for control experiments would eliminate all background problems due to binding of endogenous Erb2 which is normally expressed on the surface of mammalian cells.

Three phage display libraries were used for these studies, that were purchased from New England Biolabs. Each of these libraries was subjecetd to several cycles of binding, saline washing, elution at acidic pH, and amplification using (a) Erb2-overexpressing versus control SF9 cells; (b) Erb2-overexpressing versus control NIH-3T3 cells; (c) Erb2 overexpressing versus non-expressing mammary carcinoma cell lines. The phage species recovered at the end of these cycles were cloned, purified and tested for selectivity of binding to Erb2 overexpressing versus control cells.

The first library that was used consisted of a random strech of 7 amino acids expressed at the amino terminus of the phage gene III protein. At the end of 6 selection cycles, we analyzed 20 independent phages by nucleotide sequencing and found 12 that contained the same peptide sequence. These phages were found to bind to Erb2 overexpressing mammalian cells ~20 times more than to control cells. However, when the peptide encoded by these phages was synthesized and tested for specificity of binding, it was found to bind to a similar extent to both Erb2 overexpressing and to control cells. We also tested whether the affinity of binding of this peptide might be increased when tested in a bound form, after chemical coupling to a macromolecular carrier. However, even in this case results were essentially negative.

For this reason, we decided to abandon this peptide and look for additional ones that might have either a longer primary sequence or a constrained secondary structure. The second library that we screened consisted of phages with a radom 12 amino acid sequence inserted at the amino terminus of the gene III protein as in the previous library. At the end of 6 cycles of screening, no phages were recovered that bound specifically to Erb2 overexpressing cells.

We thus switched to a third library of phages that express a 7 amino acid random sequence flanked by a cystein at both ends, and inserted at the amino terminus of the gene III protein. After only two rounds of screening, more than 80% of the phages that were examined expressed the same peptide sequence. This peptide sequence was not found in 20 random phages that were examined from the initial

library. These phages bind more selectively to Erb2 overexpressing cells than to control cells (~10 fold difference in binding) and we are presently synthesizing the corresponding peptide for further testing.

2) Methods : High titer (~10^{11} particles/ml) phagemid library preparations were preabsorbed in PBS to unfixed control cells, and allow to bind for 10'. Supernatant was removed and added to unfixed Erb2 overexpressing cells for 20' at room temperature. Non-bound virions were removed by repeated washes with saline solution, while binding virions were recovered with a low pH solution. After each cycle, recovered phages were amplified by growth into bacteria. The phages recovered at the end of these cycles of positive and negative selection were cloned, purified and retested for their specificity of binding to Erb2 expressing versus control cells, and the sequence of their random amino acid inserts was established.

The second stage of this work involved the synthesis and purification of a peptide analogous to the specific random peptide expressed by the Erb2-binding phages. The purified peptide was labeled with a radioactive or fluorescence moiety and tested for its specificity of binding to Erb2 overexpressing versus control cells. The same peptide was also coupled to a macromolecular drug carrier. The peptide comprising a terminal cystein (incorporated during peptide synthesis) was conjugated with S-thiopyridyl groups of the carrier. The latter were introduced into the carrier structure via carrier conjugation with cystamine, with subsequent reduction of the S-S bridge followed by the thiol group reaction with dithiopyridine. The peptide coupled to the macromolecular carrier was tested on Erb2 overexpressing versus control cells as before.

3) Model carriers for drug targeting with cooperative vector molecules.

While testing phage display libraries for breast cancer-targeted peptides, we continued developing prototype cooperative carrier systems utilizing a previously developed model system based on N-formylpeptide - formylpeptide receptor interaction.
Peptides binding membrane markers of white blood cells, including the leukocyte N-formylpeptide receptor (FPR), have been found to accumulate in inflammatory regions as a result of association with white blood cells invading the inflammation site. This fact was utilized to study the behavior of cooperative polymer-based vectors comprising several peptide moieties, as compared to the analogous monomeric peptide. The objectives of this fragment of the study were: (i) prepare macromolecular preparations of varying length and comprising varying number of chemotactic peptide (N-formyl-Met-Leu-Phe-Lys) moieties per macromolecule length; (ii) determine the optimal range of peptide content; (iii) determine the molecule size range providing a most favorable blood clearance rate for agent accumulation in inflammatory areas; (iv) synthesize a model agent for inflammation scintigraphy comprising a g-emitting radionuclide and a fluorescent label; (v) characterize the biokinetics and imaging characteristics of the model preparation as compared to the radiolabeled peptide monomer.

Macromolecules comprising multiple f-Met-Leu-Phe-Lys (fMLFK) moieties and DTPA were synthesized using linear poly[hydroxymethyl-ethylene hydroxy-methyl-formal] (PHF) backbone. PHF, a biomimetic "stealth" polymer, is a base compound of Fleximer

family. An aldehyde form of PHF with broad molecular weight distribution (10 to 150 kDa) was conjugated with cystamine (spacer precursor) and fMLFK in one stage, at various molar ratios, in the presence of cyanoborohydride. The conjugates were subsequently reduced with borohydride to transform the unused aldehyde into hydroxymethyl groups and S-S bridges to mercaptogroups. The latter were derivatized with DTPA anhydride and (in some preparations) with trace amounts of fluorescein-5-maleimide. The resultant polymers were purified by gel chromatography, fractionated by SEC HPLC, and labeled with 111In via transchelation in citrate buffer. Control polymers containing DTPA but no peptide were prepared analogously. Radiolabeled peptide monomer was prepared via fMLFK derivatization with DTPA and labeling with 111In.

Pilot biokinetics and biodistribution studies were performed in normal male CD rats (n=3 per preparation) to determine the optimal ranges of peptide content and molecular weight of the polymer. Conjugates with broad molecular weight distribution were fractionated by SEC HPLC to obtain fractions with MW=10, 50 and 80 kDa (MW distributions overlapped by ca. 30% by HPLC). For the preliminary testing, conjugates containing from 0.5 to 20 % fMLFK (mol/mol monomer) were prepared. Pilot studies showed that the optimal range by conjugate content is ca. 3-10%, and the optimal dose range, with regard to clearance rate, is > 0. 1 mg/kg.

For the biodistribution studies in rabbit inflammation model, preparations containing 5% of each fMLFK and DTPA were synthesized. Non-overlapping 15 and 75 kDa fractions used in this experiment showed statistically significant difference in biokinetics. The preparations were labeled with 111In and injected iv into 2.5 kg New Zealand rabbits, n=4 per group, 50-100 μCi/animal (0.5 mg/kg total substance). Animals were normal or bearing focal bacterial inflammation induced by inoculation of E.Coli (clinical isolate) in thigh muscle. Indium-labeled PHF-DTPA and monomeric DTPA-fMLFK were used as control preparations. Images were acquired over a 20 hr. period, followed by a biodistribution study. Biodistribution data showed that, compared to the monomer DTPA-fMLFK, renal accumulation was greatly reduced (by 81% and 88% for 70 and 15 kDa preparations respectively), while accumulation in the infected muscle was reduced by only 37% and 72%. Target to normal muscle ratios were 25±10 and 14±4, compared to 33±21 for the monomer. In blood, testes, adrenals and heart, accumulation was reduced by 40-50%. Hepatic and splenic depositions were reduced by 40% for the 15 kDa preparation, and increased by 50% for the 70 kDa one. In other tissues, label content did not significantly differ from the monomer. By 20 hr., accumulation of DTPA-PHF-fMLFK in the infected site resulted in clear delineation of the inflammation in all images. It has been found that the high molecular weight preparation accumulate in the inflammation, in part, as a result of non-specific vascular leakage/retention, analogous to that in tumors. Accumulation of the low molecular weight preparation in the inflammatory site was completely target-specific, i.e., entirely dependent on the action of the chemotactic peptide. Based on this observation, we hypothesize that small targeted polymers may be preferable as targeted carriers; this hypothesis will be further tested in the ongoing research.

Conclusions

We have optimized conditions and screened three different phage display libraries for peptides that selectively bind to Erb2, an important cell surface receptor overexpressed in breast cancer cells. The first peptide sequence that was identified on an Erb2 binding phage did not retain the same specificity of binding when tested as an isolated peptide or a peptide bound to a macromolecular drug carrier. A second Erb2 binding peptide with a constrained secondary structure has also been identified on a phage, and its binding properties as an isolated peptide are currently being tested. In conclusion, we have confirmed that the phage display technology can be used to identify peptides that bind to proteins expressed on the surface of breast cancer cells. The challenge for the upcoming year will be to identify peptides that retain specificity of binding when separated from the phage and tested in a free form in solution and/or coupled to a macromolecular carrier. A model technology for producing cooperative (multivalent) vectors for drug targeting utilizing multiple peptidemoieties has been demonstrated (see enclosed reprint in the Appendix). The model macromolecules bearing multiple model peptide (fMLFK) showed dramatically reduced renal accumulation, practically unchanged deposition in RES (which has been a major concern regarding preparations of this type), and excellent accumulation in target regions. The benefits of macromolecule size and peptide content optimization has also been demonstrated. Development of multipeptide-bearing carriers, with an accent on smaller (<30 kDa) forms, will continue in the upcoming research.

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MODEL COOPERATIVE (MULTIVALENT) VECTORS FOR DRUG TARGETING

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Introduction

This study was intended to test the feasibility of drug targeting with cooperative vector molecules (macromolecules comprising multiple target-binding moieties) utilizing a chemotactic peptide model. Peptides binding membrane markers of white blood cells, e.g., leukocyte N-formylpeptide receptor (FPR) have been found to accumulate in inflammatory regions, presumably as a result of association with white blood cells invading the inflammation site.[1]

The central hypothesis of this study suggests that peptide conjugation with macromolecules opens the way to dramatic improvements in pharmacokinetics by means of (1) regulating the blood clearance via decreasing the rate of renal and, possibly, RES clearance and (2) increasing the agent-leukocyte association constant via cooperative binding effect of multiple peptide molecules exposed on the carrier. The cooperative character of agent-leukocyte interaction suggests an additional opportunity to explore a (3) hypothetical thermodynamic discriminatory effect that is expected to result in a more selective agent association with leukocytes and suppression of non-specific interactions with other tissues.

The objectives of this study were: (i) prepare macromolecular preparations of varying length and comprising varying number of peptide (N-formyl-Met-Leu-Phe-Lys) moieties per macromolecule length; (ii) determine the optimal range of peptide content; (iii) determine the molecule size range providing a most favorable blood clearance rate for agent accumulation in inflammatory areas; (iv) synthesize a model agent for inflammation scintigraphy comprising a γ-emitting radionuclide and a fluorescent label; (v) characterize the biokinetics and imaging characteristics of the model preparation as compared to the radiolabeled peptide monomer.

Methods

Macromolecules comprising multiple f-Met-Leu-Phe-Lys (fMLFK) moieties and DTPA were synthesized using linear poly[hydroxymethyl-ethylene hydroxy-methyl-formal] (PHF) backbone. PHF is a biomimetic "stealth" polymer developed in our laboratory.[2] An aldehyde form of PHF with broad molecular weight distribution (10 to 150 kDa) was produced via exhaustive periodate oxidation of poly-[1->6]-α-D-glucose (dextran B-512F). Cystamine (spacer precursor) and fMLFK were conjugated, at various molar ratios, with the aldehyde polymer in the presence of cyanoborohydride in one stage. The conjugates were subsequently reduced with borohydride to transform the unused aldehyde into hydroxymethyl groups and S-S bridges to mercaptogroups. The latter were subsequently derivatized with DTPA anhydride at pH=8 and (some preparations) with trace amounts of fluorescein-5-maleimide. The resultant polymers (Figure 1) were purified by gel chromatography, fractionated by SEC

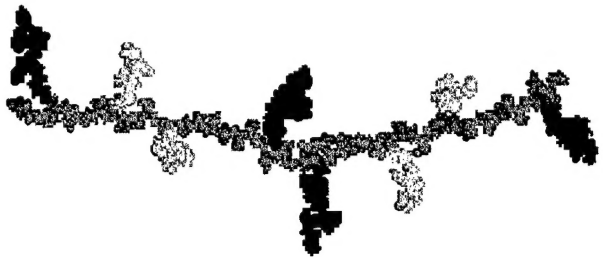

Figure 1. The structure of fMLFK-DTPA-PHF conjugate. The PHF backbone (ca. 1 kDa fragment shown) is modified by fMLFK (black) and DTPA (light gray) at random positions.

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HPLC, and labeled with ^{111}In via transchelation in 0.2M citrate buffer, pH=5.6.

Control polymers containing DTPA but no peptide were prepared analogously. Radiolabeled peptide monomer was prepared via fMLFK derivatization with DTPA and labeling with ^{111}In.

Pilot biokinetics and biodistribution studies were performed in normal male CD rats to determine the optimal ranges of peptide content and molecular weight of the polymer.

The optimized 15 kDa and 70 kDa preparations comprising fMLFK and DTPA (5% of each, mol/mol monomer) were labeled with ^{111}In and injected iv into 2.5 kg New Zealand rabbits, n=4 per group, 50-100 μCi/animal (0.5 mg/kg total substance). Animals were normal or bearing focal bacterial inflammation induced by inoculation of E.Coli (clinical isolate) in thigh muscle. In-labeled PHF-DTPA and monomeric DTPA-fMLFK were used as control preparations. Images were acquired over a 20 hr. period, followed by a biodistribution study.

Results

Conjugates prepared on the basis of the aldehyde-polymer with broad molecular weight distribution were fractionated by SEC HPLC to obtain fractions with MW=10, 50 and 80 kDa (MW distributions overlapped by ca. 30% by HPLC).

For the preliminary testing, conjugates containing from 0.5 to 20 % fMLFK (mol/mol monomer) were prepared. Pilot studies in rats (n=3 per preparation) suggested that the optimal range by conjugate content is ca. 3-10%, and the optimal dose range, with regard to clearance rate, is > 0. 1 mg/kg.

For the biodistribution studies in rabbit inflammation model, preparations containing 5% of each fMLFK and DTPA were synthesized. Non-overlapping 15 and 75 kDa fractions used in this experiment showed statistically significant difference in biokinetics.

By 20 hr., accumulation of DTPA-PHF-fMLFK in the infected site resulted in clear delineation of the inflammation in all images.

Biodistribution studies showed that, compared to the monomer DTPA-fMLFK, renal accumulation was greatly reduced (by 81% and 88% for 70 and 15 kDa preparations respectively), while accumulation in the infected muscle was reduced by only 37% and 72%. Target to normal muscle ratios were 25±10 and 14±4, compared to 33±21 for the monomer. In blood, testes, adrenals and heart, accumulation was reduced by 40-50%. Hepatic and splenic depositions were reduced by 40% for the 15 kDa preparation, and increased by 50% for the 70 kDa one. In other tissues, label content did not significantly differ from the monomer.

Discussion

The concept of this study suggests modification small vector molecule pharmacokinetics via modulation of the in vivo transfer and utilization of thermodynamic cooperative binding and discrimination effects in macromolecule interactions with tissues. Model macromolecules bearing multiple fMLFK demonstrated dramatically reduced renal accumulation, practically unchanged deposition in RES (which has been a major concern regarding preparations of this type), and excellent accumulation in inflammatory regions, which demonstrates the potential benefits of the approach. Optimization of macromolecule size and peptide composition/content can be expected to provide, in this particular case, preparations with superb inflammation targeting capabilities. The approach can be further extended to other peptides and various ligands that are target-specific, even if their binding affinity is insufficient for conventional targeting.

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